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Symposium Director

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PREFACE

A symposium on Biochemistry of Morphogenesis was held (September 1st to 4th, 1958) as part of the IVth International Congress for Biochemistry in Vienna—this volume represents the Proceedings of that Symposium. In recent years there has been achieved an increasing degree of convergence in studies (by morphologists and biochemists alike) at the molecular aggregate level. It may well appear in the near future that the study of morphogenesis is, in reality, an approach to the study of macromolecular biochemistry. Recent advances in our knowledge of protein synthesis, adaptive enzyme formation, and polysaccharide–protein aggregates are of general interest and bear upon most problems in morphogenesis. And, one need not emphasize, study of the mechanism of gene–enzyme interaction is a problem of the first magnitude, and of paramount concern alike to those studying morphogenesis, genetics and biochemistry. Although a symposium on biochemistry of morphogenesis had not previously been held as part of an International Congress for Biochemistry and, though relatively few biochemists may recognize the area as one currently ready for exploration, it is likely that this area will, in the very near future, become a scene of intense biochemical interest.

The Symposium was devoted to consideration of morphogenesis in its broadest sense—equal attention was given to plant, animal and microbial systems in which attempts had been made to explore the molecular basis of cellular form. The four sessions of the Symposium were organized under the following topics:

Biochemical Control of Differentiation in Isolated Cellular Systems

Regulation of Differentiation in the Developing Organism

Protein Synthesis and Cellular Differentiation

Experimental Systems for the Study of Biochemistry of Morphogenesis.

One might well imagine that the different connotations of the terms *morphogenesis*, *development*, and *differentiation* to experimental embryologists, plant physiologists, microbiologists, and biochemists would preclude satisfactory communication, but the common denominators among cellular architecture, at the molecular level, proved to be very efficient "noise-suppressors" and a maximum of "signal" came through. One may say, the language of biochemistry hurdles barriers to communication among those interested in morphogenesis of plants, animals or microorganisms.

Appreciation is expressed for the grant for this Symposium that was awarded by the National Advisory Health Council, and the Morphology and Genetics Study Section, of the United States Public Health Service, and which was administered by the American Institute of Biological Sciences.

WALTER J. NICKERSON

New Brunswick, N.J.
October, 1958

CONTENTS

BIOCHEMICAL INDICES OF EMBRYONIC DIFFERENTIATION

by SØREN LØVTRUP

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Embryonic material has been of enormous importance for the study of the mechanism of differentiation; in no other case has nature provided material which from a uniform beginning in a short time develops into several different organs, representing a large number of different types of cells and tissues with specific chemical and morphological characteristics.

In the study of embryonic differentiation embryos of many different species have been employed, notably those of echinoderm, amphibian, avian and mammalian species. In the present contribution I have chosen to deal with results obtained on amphibians, my own field, because I know that there are people present here who are much more competent to discuss the results obtained with species of the other groups. Furthermore, I want to emphasize that I do not consider it my task to discuss results in great detail, but rather to try to present more generally the information we have obtained from the study of chemical differentiation in embryos.

The concept of "embryonic differentiation" was originally established by morphologists, consequently it comprised only those parts of the differentiation process which can be observed directly. It is not all phases of the differentiation process which are visible to the eye. Thus, as a result of the work of experimental embryology it has been possible to distinguish between two phases of differentiation (cf. Huxley[1]). The first one is called the primary, invisible, intrinsic (or, in a less fortunate expression, the chemical) differentiation; during this phase the presumptive fate of the cells is determined, although no visible morphological changes occur. During the secondary phase (the functional differentiation), the cells assume their final appearance, and the various organs and tissues begin to function. This latter phase may again be divided into two processes, cytodifferentiation and tissue elaboration. Experimentally these may be separated in work with explants of embryos in tissue cultures.

Many different definitions of "embryonic differentiation" exist. It is not my intention to add to the confusion, but yet it might be worth while to try to define this process from a chemical point of view. In doing this,

it will be easier for us to decide which chemical events are part of the differentiation process, and which are not. Chemically, embryonic differentiation is a unidirectional chain of events, in which various metabolic patterns are elaborated and distributed among different types of cells, in such a way that each type acquires a limited number of specialized functions. As far as the irreversibility is concerned, it should be mentioned that this only holds as long as the cells remain part of the whole organism. In cell cultures certain of the specialized functions are lost, reversibly it is believed. For this "reversible" part of the differentiation process the word "modulation" has been introduced (cf. Weiss[2]).

The differentiation process as defined here comprises only such substances as are integral components of the organization of living cells, thus including the chemical mechanisms of the specialized functions, but not substances like inorganic salts, energy reserves, excretion products, etc. These cannot be considered part of the differentiation process proper, although they may be subject to influences by it.

The biochemical study of embryonic differentiation has several aims. The foremost is probably to correlate the chemical activities in the developing embryo with the morphological changes which have been so thoroughly described in the classical embryology. The use of chemical parameters in this context of course goes beyond the morphological description in so far as it, besides the qualitative aspects, also permits a characterization of the differentiation process in quantitative terms. From this point of view, it may be very fruitful to study the functional differentiation in intact embryos. Otherwise, it seems that this process is better studied on cultures of isolated explants. Another more difficult task is to try to determine the chemical nature of those morphologically invisible differentiation processes which occur during early embryogenesis. The greatest challenge to those working in this field lies perhaps in the development of experimental chemical embryology. This work is promising, because the use of chemical methods may make possible the evaluation of the influence of changes in the experimental conditions which are too slight to produce any qualitative effects.

The changes in content during embryogenesis have been determined for a large number of substances. It seems that in principle the only condition required for a substance to be included is that it is found in the egg, and that a reasonably simple method exists for determination of it. The substances which have been investigated may be divided into two groups. The first comprises inorganic salts, carbohydrates, fat, total and protein nitrogen, amino acids, lactic acid, ammonia, urea, etc., i.e. the kind of substances which we do not consider as parts of the differentiation process proper. It is true that total and protein nitrogen, and amino acids, represent the substances of the differentiation proper, but such determin-

ations are too crude to supply insight into the mechanism of differentiation, above all because they do not distinguish between reserve material and active protoplasmic components.

The substances in the second group are notably proteins with specific properties (antigenic, catalytic, etc.), nucleic acids, and various coenzymes. In the present paper all stress will be laid upon enzymes and nucleic acids. Antigens have advantages in certain aspects; they may thus be tissue specific (e.g. lens protein, cf. Ten Cate and Van Doorenmaalen[8]), but otherwise the study of these proteins does not seem to yield information different from what is obtained from the study of enzymes. For comparison of the results obtained in different stages of development it is necessary to choose a reference basis. If we extend our work throughout the entire development, the only constant quantity is the individual. We therefore have to give our results as content or activity per embryo. If we wish to follow the differentiation of individual organs, we must wait until the organ primordia have become separate entities. After this stage we can follow the changes in content or activity per organ.

We are now ready to evaluate the results obtained by the study of embryonic differentiation. The changes in a certain chemical substance are followed during the development, and a curve is plotted representing the relative change as a function of time (or rather of morphological development). If this is repeated with another substance, a different curve is almost surely obtained, and if so, we are entitled to state that in chemical terms a differentiation has occurred, i.e. the chemical composition with respect to the measured parameters has changed during development.

When the changes in a number of different substances have been determined in this way, a pattern of differentiation is obtained. This pattern may now be compared with the morphological changes known to occur simultaneously. For this purpose there are three features of the curves to take into consideration: the maximum relative increase, the time at which the increase begins, and the shape of the curves, i.e. possible breaks, etc.

It was mentioned that the present discussion will deal with enzymes and nucleic acids. We will begin the presentation of experimental results with the latter, i.e. DNA and RNA. As far as the DNA is concerned, it is possible to maintain the opinion that to some extent changes in this substance do not represent differentiation. According to current views it is identical with respect to both composition and amount in all cells (cf. Vendrely[4]). This statement disregards the existence of cells with polyploid nuclei, which seems justified, partly because the occurrence of polyploidy is relatively infrequent, except in certain organs such as the liver, and partly because the nucleo-cytoplasmic ratio seems to be the same for diploid and polyploid cells. The amount of DNA is thus not a

true measure of the actual number of cells, but it represents the number of cells if all were diploid, and this quantity is even more useful from the chemical point of view. The importance of DNA determinations lies in that they yield information about the mitotic activity in the developing embryo. It is useful to know about this as a background for the remaining chemical activity in the embryo. One might choose to use DNA as a reference for the other chemical processes, in this way obtaining values proportional to the content or activity per cell. As the relative increase for DNA is much higher than for any other substance measured hitherto, one would obtain a series of decreasing curves, which might give a misleading impression of the chemical activity during embryogenesis. Furthermore, the comparison of individual curves of the differentiation pattern would be more difficult, especially because the synthesis of DNA occurs with a certain rhythm, and the individuality of the various curves would be blurred by having this rhythm superimposed upon their own.

In the fertilized amphibian egg there is an amount of DNA corresponding to about 3–5000 nuclei. This DNA is mainly localized in the cytoplasm.[5] No increase in DNA can be demonstrated during segmentation.[5–8] Since the number of cells in the late blastula is of this order (3500 for *Bufo cognatus*; cf. Bragg[9]), it is reasonable to assume that the DNA is a reserve available for the first very fast cell divisions during segmentation.

As far as the further development is concerned, the DNA synthesis can be divided into three phases, one occurring during gastrulation, but independently of this latter process as seen from the fact that it occurs in lethal hybrids (Fig. 1). This phase mainly represents the cell divisions

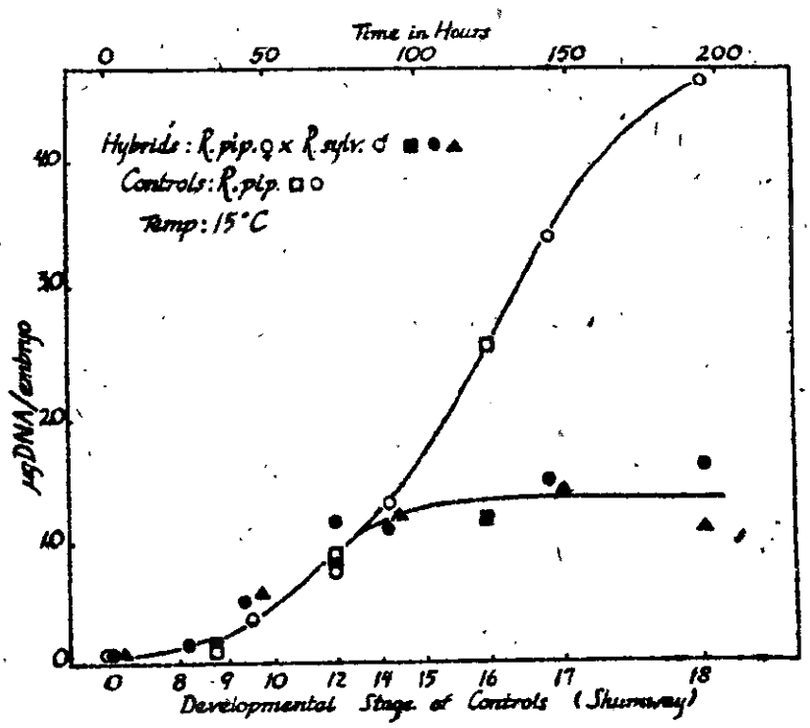

Fig. 1. Deoxyribonucleic acid content of normal and hybrid embryos at various stages of development.[8]

occurring in the blastoporal region, i.e. the material which in the normal embryo invaginates. Whereas DNA increases more or less exponentially during this first phase, the second is linear, following directly after the first one in normal embryos, but not in hybrids.[8] The second phase of DNA synthesis occurs during the neurula and early tailbud stages, and must to a considerable degree be associated with neuroectodern development. DNA synthesis stops almost completely at some late tailbud stage, to be resumed only some time during larval development when the third phase of DNA synthesis begins (Fig. 2). This phase is certainly to some extent, but not necessarily exclusively, associated with endoderm development. The total relative increase in DNA during prefeeding development is about 1000 in the axolotl.

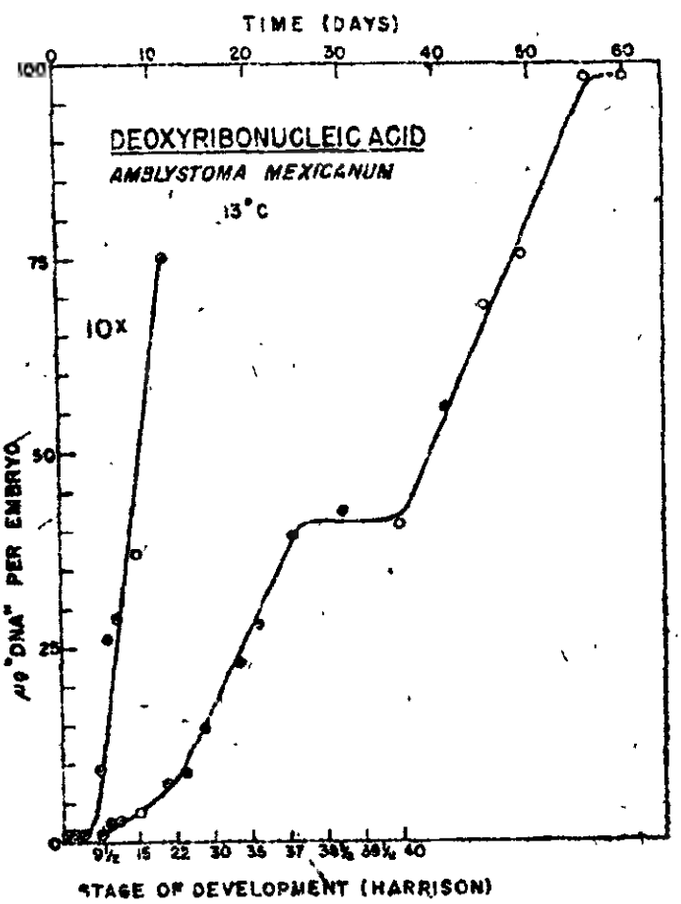

Fig. 2. Deoxyribonucleic acid content of normal embryos at various stages of development. The early part of the curve has been drawn in a 10 times increased scale.[7]

From the point of view of embryonic differentiation RNA ought to be more interesting than DNA, because the former has been shown to be specific in composition for various tissues (cf. Magasanik[10]), However, the work done so far has been devoted to establishing only the changes in the total amount of RNA. As far as this is concerned, the relative increase in RNA is quite low, the highest values observed showing only about a five-fold increase during the whole prefeeding development. The increase begins during gastrulation or neurulation; because of the low relative

increase it is very difficult to establish the exact stage.[7,11–14] There is reason to believe that the chemical composition of the RNA in the cells is influenced both by maternal and paternal genes. If this is true, we are almost forced to believe that an essential fraction of the relatively large amount of RNA in the unfertilized egg is some form of "inactive" reserve which gradually is transformed into the various specific types of RNA. The possible existence of such reserves presents a danger in the interpretation of chemical analyses.

A very complicated pattern of differentiation has been obtained from the results of enzyme determinations. As far as the relative increase is concerned, it is in agreement with expectations that those enzymes which, so to speak, represent specialized functions, such as acetylcholinesterase and apyrase (representing essentially nerve and muscle function, respectively) show the highest relative increases. Much lower increases are found with such enzymes as succinic dehydrogenase and cytochrome oxidase. It is interesting to note that enzymes like catalase and dipeptidase show very low relative increases.

The next two features, the time at which the increase begins, and the shape of the curves, may be treated together. These reflect the fact that development occurs in definite phases. This is quite well known from the morphological study of embryogenesis, but seems quite often to have been forgotten by workers in the field of chemical embryology. As a result of efforts to fit the curves into an exponential grid the discontinuity of development is quite often overlooked. An interesting observation illustrates this point. When acetylcholinesterase is determined in whole embryos, the curve representing the increase is more or less exponential.[15] However, when the increase is determined separately in fore-, mid- and hindbrain three sigmoid curves are obtained.[16] The brain acetylcholinesterase does not of course represent the total, but yet a considerable fraction of the enzyme content of the embryo.

As far as the phases of embryonic development are concerned, we have already seen that there are three distinct phases with respect to cell divisions. When the mitotic activity in a certain region comes to a standstill, a chain of chemical processes is initiated. It seems that the succession in the synthetic activity is: RNA; proteins. In so far as the tissues developing from the cells in each of the three phases are different, we should expect this difference to be revealed in the time course of the synthetic activity as represented by the enzyme curves. In agreement with this the synthesis of cholinesterase, which is produced during the functional differentiation of cells from the second phase, gains speed in early larval development. Later during larval development trypsin and amylase are synthesized, and a break is observed in the curve for alkaline phosphatase. These three enzymes are all found in tissues of endodermic origin. Apyrase, which to

a large extent is found in the muscle, ought to represent tissues derived from the first phase of cell divisions. Nevertheless, the increase in this enzyme begins at about the same time as acetylcholinesterase, an enzyme which, as mentioned, represents the second phase of cell divisions. However, it should be recalled that it is only the notochordal cells which cease to divide and begin differentiation immediately after invagination; the mesoderm cells continue to divide for some time, although at reduced rate.[9] It is reasonable to believe that the translocation of the cells to the interior of the egg slows down the rate of the differentiation process, maybe because of a limited supply of oxygen. It is not yet possible to correlate in this way all the observations concerning enzyme synthesis. It may be mentioned that alkaline phosphatase is synthesized during most of the embryonic development, but it is possible to divide the synthesis into phases, and it seems certain that it is not the same enzyme which is produced in all phases.[7] Alkaline phosphatase has also been determined in *Xenopus laevis* and *Siredon mexicanum* by Krugelis[17], in *Amblystoma punctatum* by Krugelis *et al.*[14] and in *Bufo vulgaris* by De Cesaris Coromaldi[18].

Such enzymes as cytochrome oxidase and succinic dehydrogenase are probably synthesized during early embryogenesis, but the rate is considerably increased when larval development begins.[19,20]

In Figs. 3 and 4 have been plotted a number of results obtained by

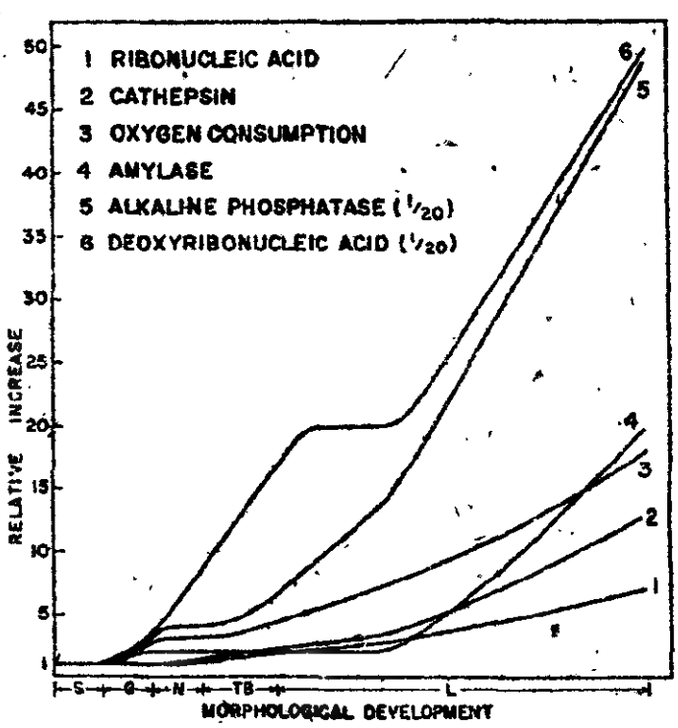

Fig. 3. Schematic representation of the chemical changes during amphibian embryogenesis. This graph only includes those substances which are synthesized to a notable extent before larval development. All curves were obtained on the axolotl, *Siredon mexicanum*.[7] The numbers in brackets indicate the factor by which the relative increase has been reduced in the graph.

determinations of nucleic acids and enzymes. It should be mentioned that none of the results obtained by Urbani and his collaborators have been incorporated (cf. Urbani[21]). This work has been carried out with *Rana esculenta*, *Rana dalmatina*, *Bufo vulgaris* and *Bufo viridis*. Determinations have been made of RNA, cathepsin, trypsin, various di- and tripeptidases, amylase and lipase. As far as RNA and the proteolytic enzymes are concerned, there is complete agreement with the results presented in the graphs here. The amylase studied by Urbani is β-amylase (the one represented in Fig. 3 is α-amylase) and this enzyme is peculiar in showing a steady decrease during prelarval development. During late larval development a steep increase was observed, just as for α-amylase. For lipase a slight increase was observed during the tailbud stages, followed by a very steep linear increase during the entire prefeeding development in the two frog species and in *Bufo viridis*. In *Bufo vulgaris* a different course was observed. Here the enzyme activity was observed to increase until the circulation was established, after which a slight decrease was observed, followed later by the same steep increase as in the other species.

The observations of Urbani on β-amylase and lipase bring up the question about decreases in the content of enzymes during embryogenesis. Generally one observes that the enzyme activities reach a maximum during late larval development (cf. Løvtrup[7,22], Urbani[21]). The decrease following the maximum is unquestionably an indication that all energy reserves

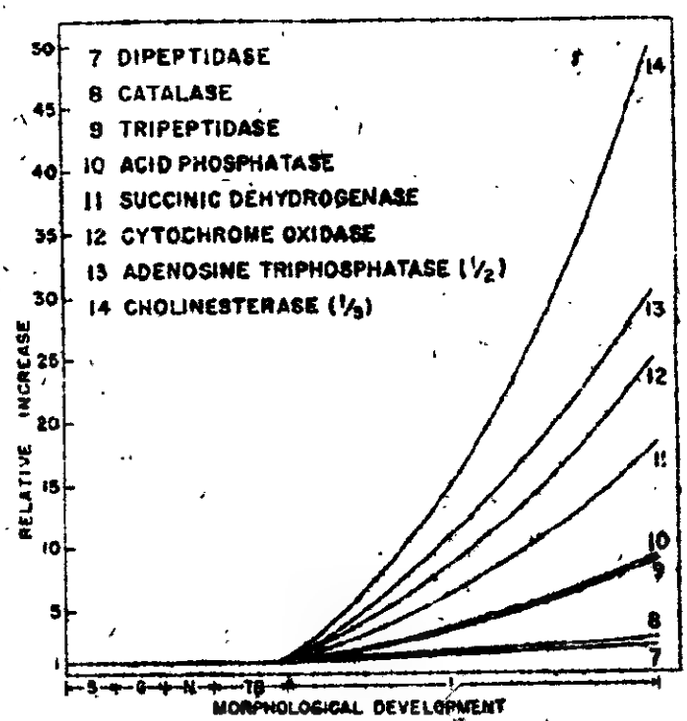

Fig. 4. Schematic representation of the chemical changes during amphibian embryogenesis. This graph includes all substances which remain essentially constant until the beginning of larval development. The following curves were obtained on *Siredon mexicanum:* 7,[23] 9,[7,22] 10,[7] 11,[20] 12[19] and 14.[16] Curve 8 was obtained on *Bufo vulgaris*[20] and curve 13 on *Rana pipiens*.[21] The numbers in brackets indicate the factor by which the relative increase has been reduced in the graph.

have been exhausted, and that the embryos are beginning to utilize the cellular proteins for combustion (cf. Løvtrup[22,23,24]). This explanation can hardly hold for the decreases observed by Urbani, but it does not seem easy to advance reasonable suggestions to explain these observations (cf. below).

Owing to the fact that we can only measure enzyme activities, but not determine the actual amount of enzyme protein, it has been suggested that the increase in enzyme activity may just reflect a transformation of enzyme from an inactive to an active form, or the removal of inhibitors (cf. the discussion by Urbani[21]). It cannot be excluded that these possibilities exist, but yet it seems that such views tend to classify enzyme proteins in a group different from other proteins. Nobody questions that myosin and other structural proteins, which can be demonstrated microscopically, or antigenic proteins, which can be measured quantitatively, are synthesized *de novo*. *A priori* there seems to be no reason to believe that the same should not hold for enzyme proteins. Furthermore, it would probably generally be agreed that the paternal genes may influence the characteristics of various enzymes, and it is difficult to see how this would be possible if all enzymes were present already in the fertilized egg.

Against this, two arguments may be raised, the first being that many enzymes are present in the egg. This is true, and these enzymes, which are necessary for the early embryogenesis, must of course possess the properties of the maternal enzymes. It may be that the decrease found in the activity of β-amylase reflects that this enzyme, being foreign to the new individual, is gradually broken down, later to be substituted by an enzyme synthesized by the embryo itself. A similar phenomenon may occur also for other enzymes, although possibly unnoticed, either because the rate of breakdown is slow or because it is masked by the simultaneous synthesis of new enzymes.

The other objection is that enzymes may be present to cover the whole prefeeding development, and that the *de novo* synthesis only begins after this stage. This argument is not very plausible either: if synthesis begins some time, then it is most reasonable to believe that it begins as soon as the organization and the synthetic capacities of the embryo are realized.

It will be realized that these questions are very difficult to attack experimentally. It seems reasonable to assume that the egg is equipped with just those enzymes which are involved in the metabolic processes occurring during the early stages of embryonic development. The enzymes involved in more specialized functions can hardly play any role at this time. It is on the other hand difficult, if not impossible, to state with certainty that an enzyme is absent. It was mentioned above that an enzyme like acetylcholinesterase shows a very high relative increase, indicating a very low

concentration in the egg. There is no doubt that the increase reflects the development of the nervous system, but is the enzyme present during the early stages really "nerve cell" cholinesterase, or is it another enzyme with similar activity? Likewise it must be admitted that negative results of enzyme determinations are not conclusive. Thus, it is quite impossible to detect any tryptic activity until the development of the gut commences, but we cannot conclude that there is no trypsin present, only that the activity is too low to be measured with the available methods.

It is seen that this antagonism between preformism and epigenesis on the chemical level is quite hard to tackle; and it is, of course, out of place to enter upon a more detailed discussion on this occasion; let it suffice to say that when the morphologists have finally buried the ghost of preformism, it seems very unfortunate indeed if the chemists try to revive it.

From the presentation given above it is seen that the work on the biochemistry of embryological differentiation has been rather successful with respect to the correlation between the morphological and the chemical differentiation. As far as the primary differentiation is concerned, the situation is different. We do not as yet know anything about what goes on during this phase of development. Some of the synthetic activity observed during embryogenesis may be associated with the primary differentiation, but owing to our lack of knowledge it is impossible to demonstrate any causal connections.

With respect to experimental chemical embryology, very little work has been done in this field as yet. As illustrations of work of this kind may be mentioned the comparison of phosphoprotein phosphatase in normal, haploid and lethal hybrid embryos,[25] and the previously mentioned determinations of DNA-synthesis in lethal hybrids.[8] The experiments dealing with the influence of temperature on synthesis of enzymes also belong in this category. This effect has been investigated for alkaline phosphatase in *Amblystoma punctatum*,[14] for di- and tripeptidase, in *Siredon mexicanum*,[32] and for lipase in *Bufo vulgaris* (Urbani Mistruzzi; cf. Urbani[31]). For the peptidases it was found that high temperatures lead to a decreased maximum content of enzyme, and the same was found for lipase at low temperatures. The effect of high temperatures is almost certainly caused by the precocious combustion of protein (cf. Løvtrup[24]), and the same cause is probable at low temperatures.

In the preceding discussion the curves representing the synthetic activity of developing amphibian embryos have been taken to represent differentiation. On this point there is no general agreement among embryologists, in so far as some of these curves are often taken to represent the growth of the embryos. We therefore have to discuss the justification for the point of view adopted here.

It cannot be questioned that some process deserving the designation of growth may occur at some stage during embryogenesis. The greatest difficulty in assessing this process lies in the fact that it is overshadowed by the differentiation processes occurring simultaneously. Thus, when we see a number of rising curves, representing chemical changes during embryogenesis, the question is immediately raised: How much of this increase represents embryonic growth, and how much differentiation? And if we have not tried to clear our mind with respect to the distinction between growth and differentiation, we are completely lost when trying to answer this question.

To illustrate the point let us consider that we are dealing with determination of a particular enzyme, say alkaline phosphatase. If this enzyme is synthesized by a cell which before contained none or only a slight amount of this enzyme, then we are definitely dealing with a differentiation process. If the enzyme content of the cell is doubled or at least increased in connection with a cell division, the enzyme increase may be said to represent a growth process. We cannot with our methods distinguish between the enzyme produced on the one occasion and that produced on the other.

In order to answer this question it is necessary to obtain a reasonably clear idea about what we understand by "growth".

When the problems of growth and differentiation are studied in multicellular organisms there is one very important distinction to make in order to avoid confusion. This is that we must consider these processes from at least two angles, viz. on the cellular level and on the level of the whole organism. Much ambiguity concerning the question of growth of cells stems from the fact that this is often taken to be equal to cell multiplication. This latter process may, however, occur in at least two radically different ways. In the first one, the daughter cells are identical, qualitatively and quantitatively, with the original cells. Generally the concomitant volume increase (growth) occurs before the division, but the time of this increase is immaterial in the present context. This kind of cell multiplication may be observed in cultures of micro-organisms. Also the cell divisions occurring during the post-embryonic growth phase in multicellular organisms may be considered to be of this type. Cells may, however, also divide without any associated volume increase. This kind of cell division is prevailing during the early phases of embryogenesis. Most morphologists would probably agree in stating that the former type represents a growth process, the latter not.

I believe that the concept of growth has been blurred by being used in association with reproductive processes. In the use of the word is implied that something small (animal, cell, bacteria, etc.) turns into something large of the same kind. I believe that it is important to stress that when a

small animal turns into a large one, then we are dealing with a growth process (including some differentiation, cf. below). When the volume of a cell or a bacteria increases, then we may be concerned with a reproductive process. Reproduction in animal species is also associated with a volume increase, confined as known to the one sex, but few would probably feel justified to characterize this as growth on the part of the mother organism.

If we could refrain from using the word growth on the cellular level, but instead talk of multiplication, division or reproduction, much clarity would be gained. Above all, we would be able to give a completely unambiguous definition of growth; viz.: Any system consisting of cells (cultures of micro-organisms, cell- and tissue-cultures, multicellular organisms) is said to grow when the number of cells of the system increases. The number of cells may be determined indirectly if all parameters increase proportionally (as, for instance, during the multiplication of micro-organisms), otherwise they must be counted, or estimated by DNA determinations, as suggested by Davidson and Leslie[26].

This is not the place to discuss the inconsistencies involved in the usual methods for estimating the growth of whole organisms, but a few comments may be justified. The most common indices employed are length, volume or weight. As far as the two latter are concerned, it should be mentioned that they may register "growth" a long time after the natural growth has ceased (e.g. during training, by increase in fat deposits, or by water uptake). They likewise do not exclude the phenomenon of "degrowth", which is unfortunate, because much clarity is probably gained by regarding growth as a unidirectional process.

Length is a better measure of growth, at least when applied to an organism with a rigid skeleton, because the growth in length in this case is an essentially irreversible process. The "reversibility" of growth is observed when length is used on such animals as planarian worms.

To use length as a measure of growth during embryogenesis is of course acceptable if it is understood that it serves only as an indication of the fact that development occurs. But when at one time we have a spherical egg, next an embryo in the tailbud stage, and finally a larva, then it must be realized that determination of the length is more an estimation of differentiation than of growth. From the time when the larval stage has been reached, and up to the beginning of metamorphosis, it is of course justified to use total length as an index of growth.

As mentioned, if we accept the number of cells as an index of growth, we have an unambiguous index of growth. The main question is whether we can accept the ensuing picture of the growth process which is radically different from the one we are accustomed to from the use of other indices. Before we discuss the consequences of using cell number (DNA) as a measure of growth, there are three points to be noticed. The first one is

that the remarkable metabolic "inertness" of DNA (indicating constancy of cell number), which repeatedly has been demonstrated, serves to fulfil the requirement that growth should be "irreversible". Secondly, there is no doubt that during the growth period following functional differentiation, growth in body size is preceded by cell divisions. This does not necessarily mean, however, that cell divisions (DNA synthesis) go parallel in the different organs; on the contrary, in some DNA synthesis stops very early, in others it continues for a long time (cf. Løvtrup and Swanson[27]). Finally, it should be noted that the increase in weight may continue for a considerable period after increase in cell number has ceased in a number of organs.[27] These observations do not necessarily detract from the value of DNA as a measure of growth. As far as the weight increase is concerned, we know that it mainly represents deposition of fat, calcification of the skeleton, etc., i.e. processes which hardly deserve the designation of growth. The use of DNA has disadvantages also, first of all, that it is necessary to kill the organism, secondly, that it is not easily applicable on larger organisms. In amphibian embryos, where DNA determinations can be made without great difficulties, we arrive at the following picture of the growth process. If we include all DNA, i.e. also the cytoplasmic reserves in the unsegmented egg, we will find that no growth occurs during segmentation, but if we count the number of cells the relative growth corresponds to a factor of about 3000 (cf. refs. 5–9). Actually it seems reasonable to maintain the view that no growth occurs during segmentation (cf. Weiss[2]), and in that case the conclusion derived from the DNA determinations is acceptable.

As mentioned above the DNA synthesis, and thus growth, during amphibian development occurs in three distinct phases. This unconventional conclusion may be unacceptable to those who consider growth to be always an exponential (or at least quasi-exponential) process. As far as this point is concerned, it is well established that pure growth (i.e. growth without differentiation occurring for instance in micro-organisms) is exponential. It has also been found that growth associated with only a slight amount of differentiation, such as occurs in postembryonic life, is quasi-exponential. However, there seem to be no theoretical reasons for expecting that a growth process associated with profound differentiations be exponential.

Thus, the fact that the DNA curves do not, except for the very first part, show anything resembling an exponential increase, is no reason for rejecting DNA as an index of growth. The relative increase of DNA during prefeeding development is about 1000-fold. This seems a very high value for the relative growth, but it is not possible to state whether this value is too high, or those obtained with other indices are too low. We may now turn our attention to the process of differentiation. Above we

have defined "embryonic differentiation" in chemical terms, and this definition may, with only minor changes, be applied also to the morphological aspects of this process. However, differentiation comprises many other biological phenomena. All the relative changes occurring during the whole postembryonic growth phase must be included under this heading. It was found ambiguous to talk about growth of cells, but this complication does not arise with respect to differentiation. We may say that a cell differentiates when it undergoes permanent changes in its composition. This is the kind of process we encounter in embryogenesis, when a cell through a number of stages assumes its final form and function. The permanence has to be stressed, partly because temporary changes may occur during the process of cell multiplication, and partly to exclude those reversible steps of the differentiation process to which Weiss applies the name modulation. Cell differentiation may be, and indeed very often is, associated with a volume increase. One might speak of cell growth in this connection, but it does not seem advisable, because the essential part of the process is not the change in volume, but the change in composition. Cell differentiation does of course occur also in postembryonic life, although to a lesser extent (e.g. during the sexual maturation process). In micro-organisms cell differentiation is presumably rare, but some instances may be mentioned. Thus when a cell is induced to synthesize large amounts of an enzyme which normally is absent or present in a low concentration, we are justified to call this a differentiation process. In a paper read at this symposium Zamenhof describes how treatment of *E. coli* with 5-bromouracil leads to a morphologically different cell type. In these cells the ratio of cytoplasm to DNA may be increased up to 20 times. In accordance with the definition given here we would have to conclude that the chemical treatment had caused a differentiation of the bacteria.

In general we may state that a system composed of cells undergoes differentiation, when any of the integral cells differentiate. This is not a necessary condition, however—the system may also differentiate as a result of changes in the relative proportion of the various cell types forming the system, or even by changes in the spatial distribution of the cells.

If the points of view concerning growth and differentiation presented above are accepted, we will be able to arrive at a reasonable answer to the question about the separation of growth and differentiation during embryogenesis. It was shown above that the chemical analyses have shown that embryonic development proceeds in three phases, and that within each phase there is a distinct succession in the chemical activity: DNA synthesis (cell divisions); RNA synthesis (primary differentiation?) and synthesis of specific proteins (secondary differentiation). We know that cells which have undergone functional differentiation multiply during

the growth period following the embryonic development. Whether they do during the prefeeding development is not known, but it is not improbable that cells which have exhausted their own resources may resume mitotic activity when reserve material can be supplied from the endodermal yolk reserves through the circulatory system. The chemical activity associated with these cell divisions would necessarily represent growth of the whole embryo. Anyhow, such activity can only occur rather late in embryogenesis. All chemical activity going on before this stage, except that associated with cell divisions proper, would represent the differentiation process. We are therefore justified to conclude that the curves presented above, except those for DNA, represent the differentiation occurring during amphibian embryogenesis.

There is one more point of interest in the results described that deserves to be briefly discussed. It was noted that only after the cell division have ceased do the cells begin to differentiate. This phenomenon is particularly clear as far as the two first phases are concerned. This is another demonstration of the often-observed antagonism between cell multiplication and cell differentiation (cf. the extensive discussion of this problem by Weiss[38]). In tissue-cultures one may at will subject the cells to conditions which will favour either multiplication or differentiation. In general one may say that the poorer the medium is from a nutritive point of view, the greater is the chance that differentiation may occur (cf. Willmer[39]). In the developing embryo it seems that the potentiality for cell divisions decreases gradually, while at the same time the possibility for differentiation increases. The mechanism involved is of course quite unknown.

We have arrived at the conclusion that the synthetic activity measured by various biochemical methods, up to a certain stage at least, represents embryonic differentiation. However, we must admit that whatever results we get from this approach, they are only descriptive. Correlated with the morphological description of embryogenesis they permit the following two conclusions: When a certain organ or tissue undergoes functional differentiation, the chemical substances (enzymes, etc.) characteristic for that particular organ are synthesized. Secondly, ubiquitous enzymes increase in amount during most of embryogenesis, probably to some extent proportional to the amount of active cytoplasm.

Some may find these conclusions rather discouraging, considering the amount of work which has been devoted to the problem. However, descriptive embryology was the basis for the enormous contributions which experimental embryology has made to our understanding of the mechanism of differentiation. In the same way descriptive chemical embryology the basis for the field of experimental chemical embryology. Work of this kind is yet scarce, but there is no doubt that much insight will be gained in the mechanism of differentiation from studies in this field.

REFERENCES

1. J. S. HUXLEY; *Nature, (Lond.)* **113,** 276 (1924).
2. P. WEISS. In A. K. PARPART; *The Chemistry and Physiology of Growth,* 135 Princeton, 1949.
3. G. TEN CATE and W. J. VAN DOORENMAALEN; *Kon. Nederl. Akad. Wetensch.* **53,** 894 (1950).
4. R. VENDRELY. In E. CHARGAFF and J. N. DAVIDSON; *The Nucleic Acids.* Vol. 2, 155 New York, 1955.
5. E. HOFF-JØRGENSEN and E. ZEUTHEN; *Nature (Lond.)* **169,** 245 (1952).
6. E. HOFF-JØRGENSEN. In J. A. KITCHING: *Recent Developments in Cell Physiology.* 79 London, 1954.
7. S. LØVTRUP; *C.R. Lab. Carlsberg, Sér. chim.* **29,** 261 (1955).
8. J. R. GREGG and S. LØVTRUP; *Biol. Bull.* **108,** 29 (1955).
9. A. N. BRAGG; *Z. Zellf. Mikr. Anat.* **28,** 154 (1938).
10. B. MAGASANIK; In E. CHARGAFF and J. N. DAVIDSON; *The Nucleic Acids.* Vol. 1, 373 New York, 1955.
11. J. BRACHET; *Enzymologia* **10,** 87 (1941).
12. P. B. KUTSKY; *J. Exp. Zool.* **115,** 429 (1950).
13. M. STEINERT; *Bull. Soc. Chim. Biol.* **33,** 549 (1951).
14. E. J. KRUGELIS, J. S. NICHOLAS and M. E. VOSGIAN; *J. Exp. Zool.* **121,** 489 (1952).
15. C. H. SAWYER; *J. Exp. Zool.* **92,** 1 (1943).
16. E. J. BOELL and S. C. SHÈN; *J. Exp. Zool.* **113,** 583 (1950).
17. E. J. KRUGELIS; *C.R. Lab. Carlsberg, Sér. chim.* **27,** 273 (1950).
18. L. DE CESARIS COROMALDI; *Ric. Sci.* **25,** 2323 (1955).
19. E. J. BOELL; *J. Exp. Zool.* **100,** 331 (1945).
20. E. J. BOELL; *Ann. N.Y. Acad. Sci.* **49,** 773 (1948).
21. E. URBANI; *Ist. Lombardo (Rend. Sc.)* B **92,** 69 (1957).
22. S. LØVTRUP; *C.R. Carlsberg, Sér. chim.* **28,** 426 (1953).
23. S. LØVTRUP; *C.R. Carlsberg, Sér. chim.* **28,** 371 (1953).
24. S. LØVTRUP; *C.R. Carlsberg, Sér. chim.* **28,** 700 (1953).
25. L. MEZGER-FREED; *J. Cell. Physiol.* **41,** 493 (1953).
26. J. N. DAVIDSON and I. LESLIE; *Nature (Lond.)* **165,** 49 (1950).
27. S. LØVTRUP and V. L. SWANSON; *Acta Physiol. Scand.* (1958).
28. P. WEISS; *Principles of Development,* New York, 1939.
29. E. N. WILLMER; *Tissue Culture,* 2nd Ed., London (1954).
31. A. FRIGGERI; *Bol. Soc. Ital. Biol. Sper.* **13,** 478 (1938).
30. L. G. BARTH and L. J. BARTH; *J. Exp. Zool.* **116,** 99 (1951).

DISCUSSION

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By means of various experiments it has been shown that in a rather early stage, for example in the amphibian neurula, most parts of the embryo differ in their behaviour, in that each part can only develop into one specific organ. Such an embryo contains a mosaic of specific organ-forming areas and may be indicated as an invisibly differentiated organism. This type of differentiation among cells which morphologically still appear

to be identical was called chemodifferentiation by Huxley who, in this way, left a problem for the biochemists. Furthermore, it was found that, prior to this stage, the potency for developing a certain organ resides in a territory which extends far beyond the definitive organ-forming area. Such a territory was defined by Weiss[1] as a morphogenetic, organ-forming field. The fields for various organs must overlap each other and one major problem of morphogenesis is found in the mysterious process through which the definitive organ-forming areas are segregated.

Turning to the problem of chemodifferentiation we may begin by asking the question as to how do adult organs differ from each other in their chemical composition.

When such differences have been found they may be searched for in early embryos to detect indices of chemodifferentiation. Continuing the excellent discussion of Dr. Løvtrup, we may look for organ-specificity in the field of enzymology. The occurrence of a specific type of metabolism, for example that is involved in melanin pigment formation, may be very valuable. Wilde[2] has even shown that the presence of a certain concentration of the substrate phenylalanine may induce cells to differentiate into pigment cells, although, without it, they would have developed into epidermal cells. This possibility of provoking a specific differentiation seems to present a very attractive means for the study of the differentiation mechanism itself.

Various methods for differentiating proteins are provided by immunology. It has recently been shown[3] that enzymes like alkaline phosphatase or phosphorylase, which exist in many organs, may possess organ-specific characters which have not yet been revealed by the study of their catalytic functions. Practically all proteins, after injection into animals (either alone or in combination with adjuvants), give rise to specific antibodies, and therefore immunological methods may be of general importance.

It is not surprising that most antisera, directed against a certain organ, show cross-reactions with other organs. Not only do most organs contain common components like connective tissue and blood vessels, but we know that organ cells even may take up complete proteins from blood.[4] The specificity of such antisera, containing a population of different antibodies, may be improved by absorption and precipitation of heterologous antibodies. Besides this fractionation of antibodies the antigen mixture may be separated into its various components by means of common biochemical methods. In another method of improving specificity of organ-antisera,[5] newborn rabbits are made tolerant to one or more cross-reacting organs, so that later these animals only produce antibodies to the desired organ.

Precipitation in agar media[6] enables us to count the minimum number

of antigens involved. Moreover, the Ouchterlony method is suitable to demonstrate the identity, or at least the similarity, of two antigens. Valuable results have been obtained with this method in studies on embryos of sea-urchins,[1] where at least 10 antigens were encountered in larval stages. Some of these antigens made their first appearance in gastrulae, others appeared later, and two could be identified as yolk constituents. In hybrids, paternal antigens were detected in late blastula stages, prior to the stage at which specific paternal morphological features became visible. In amphibian embryos some evidence was obtained of antigens, specific for blastula or other embryonic stages.[8] However, as in the sea-urchin experiments, entire eggs or embryos were used for immunization and these, especially in amphibia, contain a large amount of yolk in their cells, which makes interpretation of results difficult. The experiments on total embryos, giving evidence of specific transitory embryonic antigens, should also be regarded with caution, although it is well known, from the examples of haemoglobin, myoglobin and fetuin, that such antigens do exist.

The use of antisera against adult organs suffers less from these difficulties. For example, Ebert[10] showed a gradual appearance of antigens of heart, brain and spleen. Common antigens were removed by absorption. The first detectable brain antigen was found in the 9 day old chick embryo. According to Ebert, absorption with liver abolished every reaction of brain antisera. However, by using antisera against adult chick brain, obtained with the aid of adjuvants and absorbed with liver, Schalekamp and Kuyken[11] were able to show in agar plates the presence of specific brain antigens as early as in 50 hr old embryos. For one single experiment 500 embryos were used.

It is evident that, in all discussions on the first appearance of antigens in embryonic development, it should be kept in mind that all results depend largely upon the methods used: failure to detect a certain antigen may not be used as an argument in favour of its absence. This was also shown in discussions on the question whether specific antigens may be synthesized prior to, or following, and as a consequence, of morphological differentiation of the crystalline lens of the eye. Due to the absence of common factors like connective tissue and blood, this organ-part is extremely suitable for immunological research (as was shown by Uhlenhuth as early as 1903). In the chick, Burke *et al.*[8] showed the first trace of adult lens antigen to occur in 160-hr embryos. By dissecting out some 50–200 lens rudiments we[12] succeeded in obtaining a positive reaction in 60 hr chick embryos, where the lens-vesicle has not yet separated from the ectoderm; in lens-bud stages of the frog, Langman *et al.*[13] confirmed these results and, moreover, observed specific cytotoxic effects in cultures of chick embryo ectoderm and optic vesicles, taken from 20 up to 55 hr stages.

In later stages, when the precipitin test is positive, this sensitivity disappeared. No explanation can yet be given for this remarkable phenomenon.

Another route of attack is possible: instead of carefully dissecting out hundreds and hundreds of small organ rudiments, the organ-specific antigens may be separated by applying biochemical procedures to a large number of embryos. For this purpose one very potent antigen of the lens, the protein alpha-crystallin, which can be detected in millimicrogram quantities, was studied. Because of the absence of species specificity, preparations from bovine lenses could be used. The first antisera we obtained were used by Langman and by Flickinger[14] and showed higher specificity than antisera to whole lenses. However, we want to be sure that the protein preparations do not contain small quantities of other, still more potent antigens, which might confuse the results.[15] Therefore we tried to identify it in serological precipitates by means of its N-terminal amino acid, which was found to be glutamic acid. However, we subsequently found the same terminal residue to be present in other lens proteins as well, and therefore determined its C-terminal residue, which differs from those of the other lens proteins. So we hope in the near future to prove that alpha-crystallin itself really is the antigen involved in the serological reaction. The possibility of dissociating the antigen–antibody complex might be applied to precipitates which could be obtained by mixing extracts from embryonic material with antisera, whereby the specific antigen might be concentrated enormously and then identified.

It remains to be seen how far this backward tracing of antigens may go. Their synthesis may be started at various stages. It might start as early as in the oocyte, providing the embryo with minute amounts of specific building stones or specific synthesizing apparatuses.[16] Finally we should not forget that the maternal organism supplies the oocyte with various specific substances via the follicle cells, which give off large parts of their Golgi nets or even nuclear components. It is well known that serum proteins, including antibodies, are supplied to the oocyte.[17]

The study of purified protein antigens appears to offer one of the best approaches to the analysis of chemodifferentiation. Many experiments have been made on muscle proteins.[18] Very interesting results were obtained by Ebert *et al.*,[19] who used antisera to myosin and actin from adult chick heart, which, after absorption, no longer reacted with similar proteins from skeletal muscle. Myosin was detected even in 12 hr blastoderms and actin from 20 hr stages on. Most striking was the fact that in the earliest stages myosin was distributed over the entire epiblast, whereas in a later stage it became confined to the heart-forming area only. Actin, from its first appearance on, was restricted to this area.

The behaviour of myosin reminds us strongly of the development of a morphogenetic field, gradually becoming restricted to the morphogenetic

area. A somewhat similar demonstration of the evolution of a field might be deduced from Langman's observations[13] that the sensitivity to specific cytotoxic lens antisera at first resided in the entire cranial ectoderm, after which the sensitive region decreased in size and finally was restricted to the lens-forming area only, before sensitivity ceased completely. The implications of these results for the understanding of the morphogenetic mechanism are not clear. It should be emphasized that the presence of organ-specific antigens must be considered mainly as an indication of early differentiation, as a *product* of morphogenesis rather than a part of the morphogenetic mechanism itself.

REFERENCES

1. P. A. Weiss; *Arch. mikr. Anat. Entw. Mech.* **104**, 359 (1925); *Principles of Development*, New York, 1939.
2. Ch. E. Wilde, Jr.; *J. Exp. Zool.* **133**, 409 (1956).
3. W. F. Henion and E. W. Sutherland; *J. Biol. Chem.* **224**, 477 (1957).
   M. Schlamowitz; *J. Biol. Chem.* **206**, 369 (1954).
4. J. D. Ebert; *Proc. U.S. Nat. Acad. Sci.* **40**, 337 (1954).
   M. D. Francis and T. Winnick; *J. Biol. Chem.* **202**, 273 (1953).
   D. Gitlin, B. H. Landing and A. Whipple; *J. Exp. Med.* **97**, 163 (1953).
   H. N. Kent and G. O. Gey; *Proc. Soc. Exp. Biol. Med.* **94**, 205 (1957).
   J. Langman; *Proc. Kon. Ned. Akad. Wet. Amsterdam*, C 56, 6 and 17 (1953).
   C. L. Yuile, B. G. Lamson, L. L. Miller and G. H. Whipple; *J. Exp. Med.* **93**, 539 (1952).
5. F. J. Dixon and P. H. Maurer; *J. Exp. Med.* **101**, 245 (1955).
   M. Feldman and D. Yaffe; *Nature (Lond.)* **179**, 1353 (1957).
6. Ö. Ouchterlony; *Ark. Kemi Mineral. Geol.* **26**, 1 (1949).
   J. Oudin; *Ann. Inst. Pasteur* **75**, 30 and 109 (1948); In *Methods in Medical Research*, V, Chicago, 1952; *Ann. Inst. Pasteur* **89**, 531 (1955).
7. C. V. Harding, D. Harding and P. Perlmann; *Exp. Cell Res.* **6**, 202 (1954).
   P. Perlmann; *Exp. Cell Res.* **5**, 394 (1953).
   P. Perlmann and T. Gustafson; *Experientia* **4**, 481 (1946).
   P. Perlmann and J. C. Kaltenbach; *Exp. Cell Res.* **12**, 195 (1957).
8. V. Burke, N. P. Sullivan, H. Petersen and R. Weed; *J. Infect. Dis.* **74**, 225 (1944).
   R. M. Clayton; *Nature (Lond.)* **168**, 120 (1951); *J. Embryol. Exp. Morphol.* **1**, 25 (1953).
   R. S. Cooper, *J. Exp. Zool.* **101**, 143 (1946); **107**, 397 (1948); **114**, 403 (1950).
   R. A. Flickinger and G. W. Nace; *Exp. Cell Res.* **3**, 393 (1952).
   G. W. Nace; *J. Exp. Zool.* **122**, 3 (1953); *Ann. N. Y. Acad. Sci.* **60**, 1038 (1955).
   A. M. Schechtman; *J. Exp. Zool.* **105**, 329 (1947).
   I. L. Spar; *J. Exp. Zool.* **123**, 467 (1953).
   M. Spiegel; *Ann. N. Y. Acad. Sci.* **60**, 1056 (1955).

9. H. Drescher and W. Künzer; *Klin. Wschr.* **32**, 92 (1954).
I. Halbrecht and C. Klibanski; *Nature (Lond.)* **178**, 794 (1956).
H. A. Itano; *Advanc. Protein Chem.* **12**, 215 (1957).
J. H. P. Jonxis and S. K. Wadman; *Nature (Lond.)* **169**, 884 (1952).
W. M. Meyers and H. F. Deutsch; *Arch. Biochem. Biophys.* **54**, 38 (1955).
K. O. Pedersen; *J. Phys. Chem.* **51**, 164 (1948).
K. Singer, B. Angelopoulos and B. Ramot; *Blood* **10**, 479 and 987 (1955).
A. C. Allison; *Science* **122**, 640 (1955).
10. J. D. Ebert; *Physiol. Zool.* **24**, 20 (1951).
11. M. A. Schalekamp and M. P. Kuyken; Personal communication.
12. G. Ten Cate and W. J. Van Doorenmaalen; *Proc. Kon. Ned. Akad. Wet. Amsterdam*, C **53**, 894 (1950).
13. J. Langman, M. A. Schalekamp, M. P. Kuyken and R. Veen; *Acta Morphol. Neerlandico-Scandinavica* **1**, 142 (1957).
14. R. A. Flickinger, E. Levi and A. E. Smith; *Physiol. Zool.* **28**, 79 (1955).
15. M. Cohn, L. R. Wetter and H. F. Deutsch; *J. Immunol.* **61**, 283 (1949).
J. H. Vaughan and E. A. Kabat; *J. Exp. Med.* **97**, 821 (1953); *J. Immunol.* **73**, 205 (1954).
16. A. Tyler; *Growth* **10**, Suppl. 7 (1947).
P. A. Weiss; *Yale J. Biol. Med.* **19**, 235 (1947).
17. F. W. R. Brambell; *Proc. Roy. Soc.* B **214**, 113 (1926).
T. S. Painter; *Proc. U.S. Nat. Acad. Sci.* **39**, 985 (1940).
J. Schultz; *Exp. Cell Res.*, Suppl. 2, 17 (1952); *see also* E. Hoff-Jørgensen and E. Zeuthen; *Nature (Lond.)* **169**, 245 (1952); L. C. Sze; *J. Exp. Zool.* **122**, 577 (1953).
C. A. Brandly, H. E. Moses and E. L. Jungherr; *Amer. J. Vet. Res.* **7**, 333 (1946).
A. Buxton; *J. Gen. Microbiol.* **7**, 268 (1952).
R. A. Flickinger and D. E. Rounds; *Biochem. Biophys. Acta* **22**, 38 (1956).
D. T. Fraser, T. H. Jukes, H. D. Branion and K. C. Halpern; *J. Immunol.* **26**, 437 (1934).
T. Hosoda, T. Kaneko, K. Mogi and T. Abe; *Poultry Sci.* **34**, 9 (1955).
T. H. Jukes, D. T. Fraser and M. D. Orr; *J. Immunol.* **26**, 353 (1934).
M. Kaminski and J. Dubieux; *Bull. Soc. Chim. Biol.* **36**, 1037 (1954).
P. Knight and A. M. Schechtman; *J. Exp. Zool.* **125**, 271 (1954).
G. W. Nace; *J. Exp. Zool.* **122**, 423 (1953).
G. Ramon; *C.R. Soc. Biol.* **99**, 1476 (1928).
A. M. Schechtman and P. Knight; *Nature (Lond.)* **176**, 786 (1955).
H. Walter, A. Bulbenko and H. R. Mahler; *Nature (Lond.)* **178**, 1176 (1956).
H. Kirkman and G. T. Kirkman; *Nature (Lond.)* **181**, 1159 (1958).
A. L. Romanoff and A. J. Romanoff; *The Avian Egg*, p. 306, New York, 1949.
18. S. Ranzi and P. Citterio; *Exp. Cell Res.*, Suppl. 3, 287 (1955)
R. L. DeHaan; *J. Exp. Zool.* **133**, 73 (1956).
H. Holtzer, J. M. Marshall and H. Finck; *J. Biophys. Biochem. Cytol.* **3**, 705 (1957).
J. S. Johnson and Ch. A. Leone; *J. Exp. Zool.* **130**, 515 (1955).
19. J. D. Ebert; *Proc. U.S. Nat. Acad. Sci.* **39**, 333 (1953).
J. D. Ebert, R. A. Tolman, A. M. Mun and J. F. Albright; *Ann. N. Y. Acad. Sci.* **60**, 968 (1955).